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Intracellular Conditions Required for Initiation of Solvent Production by *Clostridium acetobutylicum*

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We investigated the intracellular physiological conditions associated with the induction of butanol-producing enzymes in *Clostridium acetobutylicum*. During the acidogenic phase of growth, the internal pH decreased in parallel with the decrease in the external pH, but the internal pH did not go below 5.5 throughout batch growth. Butanol was found to dissipate the proton motive force of fermenting *C. acetobutylicum* cells by decreasing the transmembrane pH gradient, whereas the membrane potential was affected only slightly. In growing cells, the switch from acid to solvent production occurred when the internal undissociated butyric acid concentration reached 13 mM and the total intracellular undissociated acid concentration (acetic plus butyric acids) was at least 40 to 45 mM. Similar values were obtained when cultures were supplemented with 50 mM butyric acid initially or when a phosphate-buffered medium was used instead of an acetate-buffered medium. To measure the induction of the enzymes involved in solvent synthesis, we determined the rates of conversion of butyrate to butanol in growing cells. The rate of butanol formation reached a maximum in the mid-solvent phase, when the butanol concentration was 50 mM. Although more solvent accumulated later, de novo enzyme synthesis decreased and then ceased.

Solvent production by *Clostridium acetobutylicum* has been studied intensely since early in this century because of the economic importance of the fermentation end products (reviewed in references 10, 13, 40, and 43). In batch cultures, this obligate anaerobe ferments sugars to butyric and acetic acids, which decrease the external pH to below 5 at the end of the exponential phase. At the stationary phase, the sugar and the preformed acids are converted to solvents (acetone, butanol, and ethanol, in the approximate ratio 2.4:6:1, as well as traces of acetoin and large amounts of CO_2 and H_2, in the ratio 0.7:24:15), thereby raising the external pH (7, 37, 42). As the stationary phase progresses, the turbidity of the suspension decreases, the cells undergo changes in morphology, the viability drops, and glucose uptake and glycolysis decrease. The ability to produce solvents seems to be associated with an early step in sporulation (23, 27, 28). Neither the mechanisms of solvent sensitivity nor those involved in the regulation of solvent production have as yet been elucidated.

Here we report that among the physiological conditions associated with the induction of butanol-producing enzymes are intracellular undissociated acid (acetic and butyric acids) concentrations of at least 40 to 45 mM and that butanol dissipates the proton motive force of growing and fermenting *C. acetobutylicum* cells.

MATERIALS AND METHODS

Growth of cells. *C. acetobutylicum* ATCC 4259 cells usually were grown anaerobically at 34°C in an acetate-buffered medium (TYA) (21). When needed, a phosphate-buffered medium (TYP) which is similar to CAB medium (26) was used. TYP medium consists of (per liter): tryptone (Difco Laboratories, Detroit, Mich.), 5.0 g; yeast extract (Difco), 5.0 g; KH_2PO_4, 0.7 g; K_2HPO_4, 0.7 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.015 g; $MnSO_4 \cdot H_2O$, 0.1 g; NaCl, 0.1 g; resazurin, 1.0 mg; and L-asparagine, 0.5 g. Separately deoxygenated and autoclaved glucose was added to a final concentration of 6% (wt/vol). Preparation of the media, inoculation, and anaerobic growth methods have been described previously (21).

Measurement of the proton motive force. The transmembrane pH gradient (ΔpH) and the transmembrane electrical gradient ($\Delta\psi$) were determined from the distribution of [7-^{14}C]benzoate (final concentration, 9 μM; 19 Ci/mol) and [^{3}H]tetraphenylphosphonium bromide (final concentration, 1 μM; 1 Ci/mol), respectively, as described previously (3). For assays with nongrowing cells, the cells were harvested from various phases of batch culture, washed twice with citrate-phosphate buffer (pH 5.0), resuspended in the same buffer, supplemented with 50 mM glucose, and assayed at 23°C. For assays with growing cells, samples of the cultures were removed, the ΔpH and $\Delta\psi$ probes were added, incubation was continued for the desired times, and the cells were processed within an anaerobic glove box as described previously (3).

The intracellular aqueous spaces of growing cells in their growth media were found to be 1.67 ± 0.21 μl per mg (dry weight) of cells (n = 12) in acid-phase cells (24 h of growth), 2.13 ± 0.30 μl per mg (dry weight) of cells (n = 12) in solvent-phase cells (48 h of growth), and 2.80 ± 0.50 μl per mg (dry weight) of cells (n = 12) in late-solvent-phase cells (72 h of growth) determined by using ^3H_2O to measure the total aqueous space in the cell pellets and by subtracting the extracellular space occupied by [^{3}H]polyethylene glycol (25).

Analysis of fermentation products. The fermentation products (acetate, butyrate, butanol, acetone, ethanol, and acetoin) were measured by gas chromatography. Samples (100 μl) of culture supernatant were acidified with 20 μl of 1 N HCl containing 50 mM isobutanol as the internal standard. A 2.5-μl portion of the acidified sample was injected into a gas chromatograph (Bendix Series 2600; Bendix Process Instruments Div., Ronceverte, W. Va.) equipped with a

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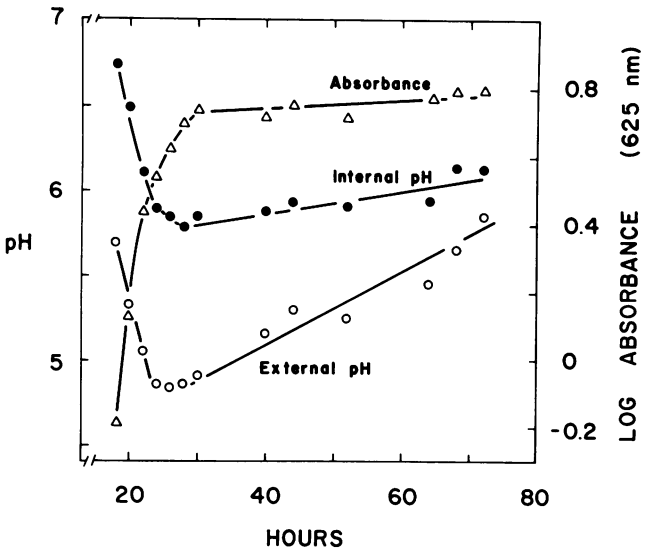

FIG. 1. Internal pH and external pH of *C. acetobutylicum* cells growing in batch culture in acetate-buffered medium. The experiments were carried out as described in the text.

flame ionization detector. The fatty acids and solvents were separated in a glass column (2 mm [inner diameter] by 2 m) packed with Chromosorb 101 (80/100 mesh; Supelco Inc., Bellefonte, Pa.) at an oven temperature of 160°C, run isothermally. The injector and detector temperature was 210°C. Nitrogen was the carrier gas.

Internal concentration of undissociated butyric acid. [^{14}C]butyrate (final concentration, 13 μM; 13.4 Ci/mol) was added to growing cells. The internal [^{14}C]butyrate concentration/external [^{14}C]butyrate concentration ratio was determined by the same techniques as those used for the ΔpH assays with the ΔpH probe [^{14}C]benzoate. The total internal butyric acid concentration (associated plus dissociated forms) was calculated from this ratio. The external butyrate concentration was determined by gas chromatography. The undissociated butyric acid concentration was calculated on the basis of a pK_a of 4.82 and was considered to be equal inside and outside the cells, since the unionized form of the acid permeates freely across cell membranes. The internal pH was calculated from the distribution of [^{14}C]benzoic acid.

Rate of in vivo butanol formation. [^{14}C]butyrate, at the concentration and specific activity described above, was added at various times to cells in batch culture. The cells were separated from the growth media by centrifugation through silicone oil after 15 and 30 min of further incubation. The supernatants were pooled, and 1.0-ml aliquots were percolated through columns (0.5 by 8.0 cm) of the anion-exchange resin Dowex 1-X8 (formate form; J. T. Baker Chemical Co., Phillipsburg, N.J.). The column was washed with 4.0 ml of water to remove nonionic butanol, and butyrate was eluted with 5.0 ml of 1 N formic acid. Fractions (1.0 ml) were collected for counting of radioactivity. The rate of butanol formation was calculated from the butyrate and butanol concentrations as determined by gas chromatography and the radioactivity in the water eluates from the Dowex 1-X8 column.

Materials. The radioactive chemicals were bought from Dupont-New England Nuclear Corp., Boston, Mass. The other chemicals were of reagent grade and are commercially available.

RESULTS

Intracellular pH of growing *C. acetobutylicum*. During typical batch growth of *C. acetobutylicum* in TYA medium, the external pH of the culture decreased from 6.0 to 4.6 (after 24 h of incubation) because of the acetic and butyric acids produced by the cells (Fig. 1). During the acidogenic phase, a fairly constant ΔpH of 0.9 to 1.1 was maintained by the cells. Thus, the intracellular pH was always more alkaline than the medium pH, but it declined in parallel to the drop in the external pH. A decline in the internal pH as a result of the production of weak organic acids has been reported previously in various clostridia (3, 5, 14, 20, 39). As cell growth reached the stationary phase and solvent production started (data not shown), consumption of the acids caused the medium pH to increase to approximately 6.0 (after 96 h); at this time, a toxic level of approximately 1.5% (vol/vol) (164 mM) butanol was reached. The internal pH remained fairly constant (5.8 to 6.0) during the solventogenic phase. As solvent production progressed, the ΔpH was dissipated gradually, presumably because of the accumulation of solvents, particularly butanol.

Internal concentration of undissociated butyric acid at the onset of solventogenesis. Previous reports have suggested that the concentration of undissociated butyric acid is important for the initiation of solvent production in *C. acetobutylicum*

TABLE 1. Intracellular concentration of undissociated butyric acid during batch culture of *C. acetobutylicum*

Culture medium	Time after inoculation (h)	pH		Butanol (mM)	Total butyrate (mM)		Undissociated internal butyric acid (mM)
		External	Internal		External	Internal	
Acetate-buffered	18	5.70	6.74	13.3[a]	9.0	85	1.0
	20	5.33	6.49	13.6	12.5	133	2.9
	22	5.05	6.11	13.5	15.1	103	5.6
	24	4.86	5.90	14.7[b]	28.3	161	13.4
	26	4.84	5.85	16.8	25.4	120	12.4
	28	4.86	5.79	18.0	26.4	74	12.8
	30	4.91	5.85	27.1	28.0	90	12.6
Acetate-buffered + 50 mM butyrate	24	5.52	6.36	13.6[a]	45.5	253	7.6
	26	5.29	6.11	14.5[b]	59.0	268	14.9
	28	5.18	6.00	17.5	57.8	245	17.7
	30	5.15	5.93	18.1	59.6	219	19.0
	41	5.20	5.60	29.2	55.4	82	16.3

[a] Contributed by the inoculum.
[b] Beginning of solventogenesis.

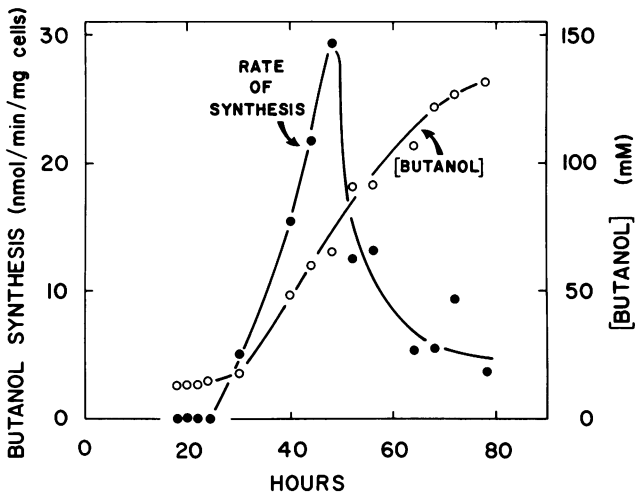

FIG. 2. Rate of in vivo butanol synthesis by *C. acetobutylicum* during batch culture. The experiments were carried out as described in the text.

(34), but this value has only been calculated. We now have measured the internal concentration of undissociated butyric acid in these cells during batch culture (Table 1). In a typical culture, solvent production started between 22 and 24 h of culture, as seen by the increase of butanol above the 13.5 mM contributed by the inoculum at zero time (data not shown). The external pH reached a minimum of 4.84 after 26 h, and the internal pH at the switch point (24 h) was 5.86 (Fig. 1). The total butyrate concentration (undissociated acid plus anion) in the medium increased during the acidogenic phase and reached a plateau of approximately 26 to 28 mM in the solventogenic phase. The total internal butyrate concentration was maximal at 160 mM after 24 h. The concentration of undissociated butyric acid inside the cells was found to be equal to that in the medium, as expected. Most important, the switch from acid to solvent production occurred when the internal undissociated butyric acid concentration reached 13 mM.

When a similar batch culture was supplemented with 50 mM butyric acid initially, the medium pH fell to 5.15 after 30 h of growth and then started to rise (Table 1). Just before solvent production was observed, the total internal butyrate concentration was greater in the butyrate-supplemented cultures than in the unsupplemented cultures (253 mM at 24 h versus 161 mM at 22 h, respectively). The internal undissociated butyric acid concentration in the supplemented cells, however, was low, 7.6 mM, at 24 h, and butanol production started when it was 14.5 mM (26 h).

When *C. acetobutylicum* cells were grown in TYP medium, which is buffered with inorganic phosphate instead of acetate, the initiation of solvent production occurred when the internal undissociated butyric acid concentration reached 18.2 mM. The undissociated acetic acid concentration in these cells was 23.7 mM, as calculated from the acetate concentration measured by gas chromatography and with a pK_a value of 4.76. Thus, the total internal undissociated acid (acetic plus butyric) concentration was 41.9 mM. This value is similar to that of cells growing in TYA medium, which contained a total internal undissociated acid concentration of 46.8 mM at the switch point, consisting of 13.6 mM undissociated butyric acid and 33.2 mM undissociated acetic acid.

Rate of in vivo butanol formation. To measure the appearance of enzymes involved in solvent synthesis, we determined the rates of conversion of butyrate to butanol in growing cells throughout the solventogenic phase (Fig. 2). The short-term rates measured in these experiments apparently were initial rates, since the amounts of [^{14}C]butanol produced from [^{14}C]butyrate in 30 min were twice those formed in 15 min (data not shown). The conversion of butyrate to butanol increased from 0 in the acidogenic phase to a maximum rate of 29 nmol/min per mg (dry weight) of cells at 48 h of incubation; after this, the rate declined to approximately one-sixth by 64 h. These in vivo rates agree with the rate of butanol accumulation in the culture. Butanol accumulated in the culture until it reached a maximum of 1.5% (vol/vol) (164 mM) after 96 h of incubation (data not shown). Consistent with enzyme induction, tetracycline (12 μg/ml) totally inhibited butanol synthesis by late-acid-phase cells (data not shown).

Effect of butanol on the proton motive force. To determine the effect of butanol on the components of the proton motive force, cells from the acidogenic and solventogenic phases were removed from the growth medium, washed, and suspended in pH 5.0 buffer supplemented with glucose (Fig. 3). In the absence of significant concentrations of solvents, acid-phase cells maintained a higher internal pH than did solvent-phase cells (6.7 versus 6.1, respectively). With increasing concentrations of butanol, the internal pH declined in cells from both phases of batch culture. At 1.5% (vol/vol) (164 mM) butanol, the maximal concentration attained in batch cultures, the internal pH was 5.4 in acid-phase cells incubated in pH 5.0 buffer and only approximately 5.2 in solvent-phase cells. A butanol concentration of 2% totally dissipated the ΔpH. Thus, the gradual collapse of the ΔpH from 0.9 to 0.3 during the solvent phase of growth (Fig. 1) can be attributed to a steady increase in the concentration of butanol.

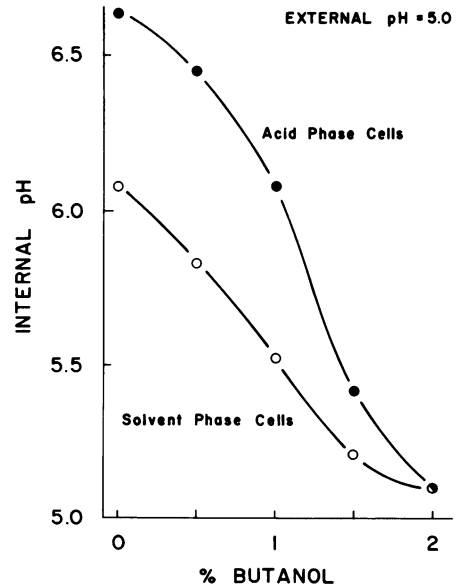

FIG. 3. Effect of butanol on the internal pH of fermenting *C. acetobutylicum*. Cells harvested from the acid and solvent phases were incubated in pH 5.0 buffer supplemented with glucose; 0 to 2% (vol/vol) butanol was added, where indicated, 15 min before the addition of the ΔpH probe, and the mixture was incubated for 15 min more. For other experimental details, see the text.

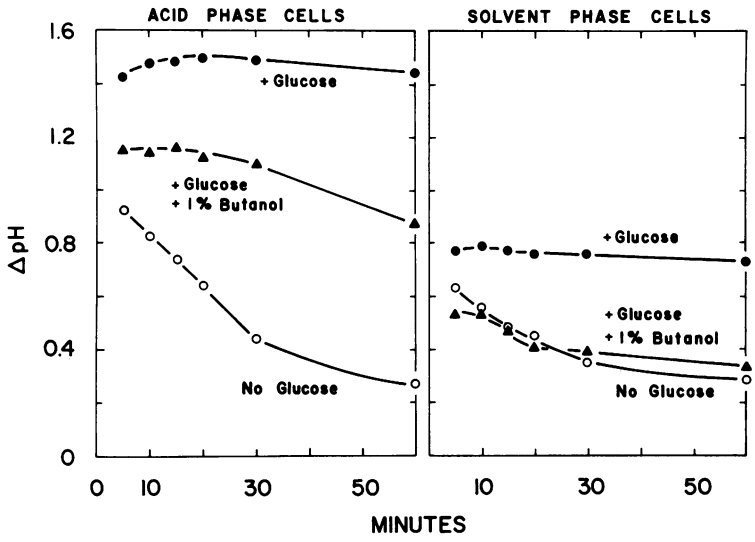

FIG. 4. Effect of energy source and butanol on the ΔpH of acid- and solvent-phase cells. The experiments were carried out as described in the legend to Fig. 3 and in the text.

Maintenance of the ΔpH requires energy. Cells assayed in buffer in the absence of an added energy substrate had a lower internal pH than did energized cells, and the ΔpH decreased over 30 min of incubation (Fig. 4). Again, acid-phase cells maintained a higher internal pH than did solvent-phase cells, and the addition of 1% butanol dissipated the ΔpH to minimal levels in cells from both phases of growth.

In contrast to the ΔpH, the membrane potential (Δψ) was affected only slightly by butanol (Fig. 5). In both acid- and solvent-phase cells, the Δψ was low (−35 to −60 mV): it was somewhat higher in the solvent-phase cells. It should be noted, however, that Δψ values of this magnitude are only approximate (24). The Δψ was relatively unaffected by an increase in the butanol concentration to 2% but was collapsed by 5% butanol (data not shown). The decline in ΔpH, then, accounted for most of the decrease in the proton motive force seen with increasing butanol concentrations. Moreover, acid- and solvent-phase cells were approximately equally sensitive to butanol.

DISCUSSION

The deleterious effects of weak organic acid end products on growth and metabolism are explained by the acidification of the interior of the cell. For example, in *C. thermoaceticum*, growth and acetate production cease when the internal pH drops below 5.5 (3). The acetic acid produced by the cells diffuses passively across the cell membrane in its undissociated form and thus acidifies the cytoplasm by ferrying H^+ across the cell membrane, thus collapsing the ΔpH. *C. thermoaceticum*, thus, requires an internal pH of at least 5.5 for growth and metabolism. A critical internal pH also has been observed in other anaerobic fermenters (reviewed in references 4 and 24). In *C. acetobutylicum*, we and others (14, 20) have shown that the internal pH decreases during the acidogenic phase because of the organic acids produced by these cells. The internal pH, however, does not go below 5.5 throughout batch growth.

In contrast, the deleterious effects of alcohols on bacterial

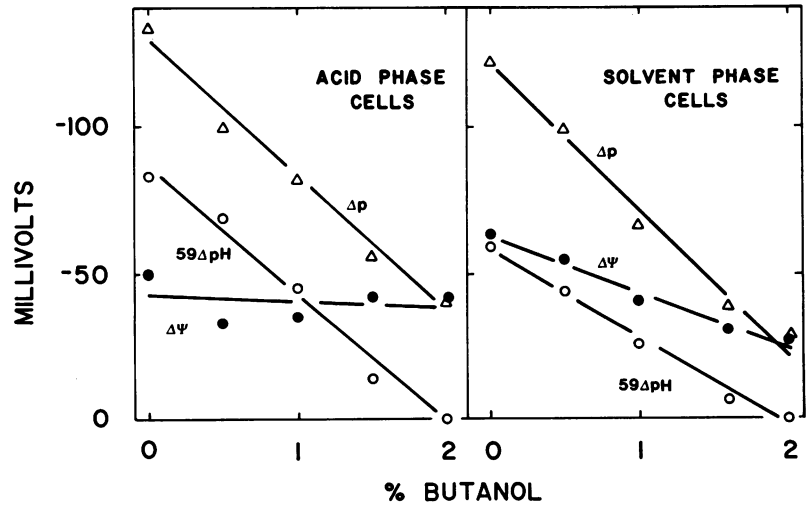

FIG. 5. Effect of butanol on the ΔpH and Δψ of fermenting *C. acetobutylicum*. The experiments were carried out at pH 5.0 as described in the legend to Fig. 3 and in the text. Δp, Proton motive force.

metabolism, although not understood (reviewed in reference 22), are most simply explained as being caused by the chaotropic effects of these compounds on the membrane structure (38). Perturbation of the orderly array of the fatty acid side chains of the phospholipids by alcohols would affect the ability of the cells to retain and exclude electrolytes and nonelectrolytes (22, 36). *C. acetobutylicum* cells treated with butanol lose phosphoenolpyruvate and sugar phosphate (21). Normally, the accumulation and exclusion of solutes is the result of active transport; for example, proton pumping by the H^+-translocating ATPase can offset the influx of H^+ ions through the perturbed membrane, but only up to a point; with more alcohol, the H^+ gradient collapses. It is possible that alcohols are toxic because they affect the functioning of membrane-embedded proteins, such as ion pumps (41), but this has not been demonstrated in clostridia. Direct effects of alcohol on the early enzyme(s) of glycolysis have been postulated for *C. thermocellum* (18). However, the condition of the cells in these experiments did not appear to be physiological; the internal pH for example, was 8.3. Moreover, the poise of the ΔpH is the net result of H^+ influx (by various H^+-linked transport systems) and efflux (by the H^+-translocating ATPase) (16). Thus, if the effect of alcohol is to impair glycolysis, and hence the rate of ATP synthesis, this would decrease the rate of H^+ efflux. However, the rate of H^+ influx could change as well; thus, measuring only the ΔpH, instead of unidirectional H^+ fluxes, cannot be used as an index of the rate of glycolysis. In addition, the $\Delta\psi$, the other component of the proton motive force (31, 32), was not considered in *C. thermocellum*.

Switching of the terminal fermentation pathways of *C. acetobutylicum* from acid to solvent production, thought to be a detoxification mechanism (22, 26), is known to include a number of physiological requirements. First, a high concentration of an energy substrate, such as glucose, is needed, suggesting that rapid catabolism is required (8, 11, 35). Second, a low medium pH (reviewed in reference 2) and an internal pH greater than 5.5 (14, 20; our data) are required. Although related clostridia produce solvents above pH 5.5 (9, 15), *C. acetobutylicum* does so only when supplemented with butyric and acetic acids (2, 12, 19, 29). Thus, it was suggested that the concentrations of internal undissociated butyric and acetic acids are important for the initiation of solventogenesis (33, 34). We have found the internal concentrations of total undissociated acetic and butyric acids to be >42 mM and that of butyric acid to be at least 13 to 14 mM.

It is known that the enzyme profiles of acid-phase cells and solvent-phase cells are different. The enzymes needed for acid production and hydrogenase, detected from their catalytic activities in cell-free extracts, decrease in solvent-phase cells (1, 17). Solvent-producing enzyme activities, in contrast, are present only in cells harvested from the solvent phase (1). We used a physiological assay for solvent synthesis, the rate of in vivo butanol formation, and showed that induction of the enzymes reaches a maximum in the mid-solvent phase, when the butanol concentration is 50 mM. Although more solvent accumulates later in batch culture, de novo enzyme synthesis decreases and then ceases.

Current hypotheses proposed for the triggering mechanism that causes the switch from acid to solvent production involve the regulation of catabolic pathways in response to the cellular concentrations of intermediates, such as butyric and acetic acids. That there is modulation is shown by the enhancement of butanol production when hydrogenase is inhibited with carbon monoxide, diverting reducing equivalents from H_2, a major electron sink, to solvent production (6, 26, 30). It was recently proposed that as butyrate and acetate reach high levels at the end of the acidogenic phase, the levels of butyryl coenzyme A and butyryl phosphate (and acetyl coenzyme A and acetyl phosphate) also increase (14). This in turn results in a relative decrease in the coenzyme A and phosphate pools, which, then, is the triggering mechanism for activating or synthesizing the enzymes for solventogenesis. The substrates (butyryl coenzyme A or butyryl phosphate and acetoacetyl coenzyme A) are available in high concentrations. In this scheme, intracellular undissociated butyric acid and acetic acid are thought not to play a role, because of their inertness. Our data, however, suggest that the uncharged forms of these acids must reach an adequate internal concentration, regardless of the total acid concentration, for initiation of solvent production to occur.

ACKNOWLEDGMENT

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